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Plaque Assay of Bovine Coronavirus in BEK-1 Cells

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(Received 8 March 1985/Accepted 20 May 1985)

ABSTRACT. The plaque formation of Japanese isolate of bovine coronavirus was studied in BEK-1 cell monolayers. The isolate readily produced clear plaques in 3 days. The plaque numbers obtained were directly proportional to the virus concentration. The procedure of plaque assay was simple, sensitive and reproducible.—KEY WORDS: BEK-1 cell, bovine coronavirus, plaque assay.

Jpn. J. Vet. Sci. 47(4): 679–681, 1985

Bovine coronavirus (BCV) has been identified as one of the causative agents of diarrhea in neonatal calves [4, 5] and adult cattle [8]. For isolation and infectivity assay of BCV, the primary cell cultures of bovine kidney have been widely used. Although some BCV strains have been adapted to cultured cells [2, 6, 9] and organ cultures of the fetal trachea [7], practical difficulties still remain in works with BCV. Inaba *et al.* [3] have found that the established BEK-1 cell line derived from bovine embryonic kidney was permissible to propagate Nebraska strain of BCV with typical cytopathic effect. Takahashi *et al.* [8], recently, isolated Kakegawa strain of BCV from adult cattle with diarrhea using primary culture of bovine kidney. The isolate was adapted in BEK-1 cells and examined for biophysical and biochemical properties [1].

The plaque assay of BCV was demonstrated in the primary and low-passaged cell cultures of the fetal thyroid and brain with trypsin treatment [6] and human adenocartinoma [9].

The present note is to describe the plaque assay method using BEK-1 cells and a BCV isolate, Kakegawa strain [1, 8], which had been plaque-cloned 3 times in BEK-1 cells by the method described below. After inoculation of the virus into BEK-1 cells and incubation at 37°C for 48 hr, the supernatant of infected culture fluid was stored at −80°C as seed virus. BEK-1 cells were grown at 37°C in Eagle's minimum essential medium (MEM) (Nissui, Japan) containing 10% newborn calf serum, 10% tryptose phosphate broth (TPB) (Difco, U.S.A.) and kanamycin (0.06 mg/m*l*). The cell monolayers were prepared in 60-mm plastic petri-dishes (Terumo, Japan) by seeding with 1×10^6 cells suspended in 5 m*l* of growth medium and incubated at 37°C for 2 days in a humidified atmosphere containing 5% CO_2.

In order to determine experimental conditions for routine assay, the kinetics of virus adsorption on the BEK-1 cells was investigated. There was no difference in virus adsorption when MEM, phosphate buffered saline (PBS:8.00 g NaCl, 0.20 g KCl, 2.89 g $Na_2HPO_4\cdot12H_2O$, 0.20 g KH_2PO_4 in 1000 m*l* distilled water, pH 7.2) or PBS containing Ca^{++} (0.1 mg/m*l* $CaCl_2$) and Mg^{++} (0.1 mg/m*l* $MgCl_2\cdot6H_2O$) was used as virus diluent. The virus was adsorbed onto BEK-1 cell monolayers at 37°C for 60, 90 and 120 min. After virus adsorption half the inoculated cultures were washed 3 times with PBS for removing the free virus. As shown in Table 1, the number of plaques was always higher in unwashed cultures than washed ones. The

Table 1. Adsorption time and plaque formation

Adsorption[a] (min)	Plaque number[b]	
	Unwashed	Washed[c]
60	121.0[d]	98.3
90	130.7	123.7
120	109.6	98.7

a) At 37°C before agar overlaying.
b) After incubation at 37°C for 72 hr.
c) 3 times with PBS after virus adsorption.
d) Average of three dishes.

Table 2. Incubation-time and plaque formation

Incubation at 37°C[a] (hr)	Plaque	
	Size	Number
48	0.8–1.4 mm	69.0[b]
72	2.0–2.8 mm	91.7
96	3.0–4.2 mm	61.0

a) After virus adsorption and agar overlaying.
b) Average of three dishes.

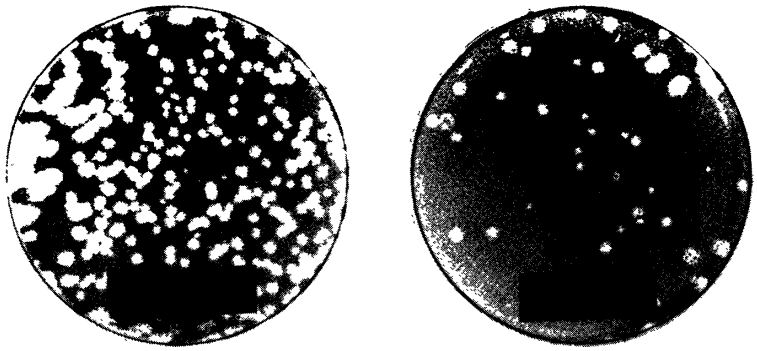

Fig. 1. Plaques produced 72 hours after inoculation with Kakegawa strain.

largest number of plaques was obtained after incubation for 90 min in both the washed and unwashed cultures. As routine procedure, it might be recommended that the inoculated cultures were overlaid without washing after virus adsorption at 37°C for 90 min.

As shown in Table 2, the inoculated cultures were incubated at 37°C for 48, 72 or 96 hr and were examined size and number of plaques. After incubation for 48 hr, clear plaques with a well-defined margin were visible without staining with neutral red. At 72 hr of incubation plaques increased in size and number, as presented in Figure 1. After incubation for 96 hr, plaques increased in size but decreased in number because of plaque fusion and degeneration of uninfected cells.

The plaque numbers obtained were directly proportional to the virus concentration, indicating that one infectious particle was sufficient to produce one plaque.

Dea *et al.* [2] and Storz *et al.* [6] reported that replication of BCV in cell cultures was enhanced with trypsin added to the medium. In these experiments infectivity titers became 5 to 10-fold higher with trypsin treatment than without trypsin. In BEK-1 cells and Kakegawa system no significant change in plaque number was seen when 1% trypsin (Difco, 1:250, U.S.A.) solution in PBS was added to the virus diluent or overlay medium at final concentration of 5 μg/ml. In contrast, plaque count increased about twice in size and number only when trypsin was added to both the diluent and overlay medium as shown in Table 3. However, the presence of trypsin in overlay medium caused severe damage of cells and plaques became ill-defined. Trypsin treatment seemed unnecessary

Table 3. Effect of trypsin on plaque formation

trypsin (5μg/ml) in		Plaque	
Diluent	Overlay medium	Size	Number
−	−	2.0–2.8 mm	31.3[a)]
+	−	2.1–2.8 mm	34.0
−	+	2.4–3.2 mm	37.7
+	+	3.0–4.5 mm	57.7

a) Average of three dishes.

for plaque assay using BEK-1 cells and Kakegawa strain.

The plaque assay method established by the results in this study was as follows: BEK-1 cell monolayers grown in dishes were washed 3 times with PBS and inoculated with 0.2 ml of the virus diluted in MEM. After virus adsorption at 37°C for 90 min, the inoculated cultures were overlaid with 5 ml of agar medium consisting of 0.9% Agar Noble (Difco, U.S.A.) and 10% TPB in MEM. The cultures were incubated at 37°C for 3 days and overlaid again with agar medium containing 0.01% neutral red. After incubation at 37°C for 6 to 8 hr, the plaque numbers were counted. Infectivity titers were expressed in plaque-forming-units (PFU).

BEK-1 cells were found to be useful for plaque assay of BCV. The method is simple, sensitive and reproducible and it might be applicable to various studies on BCV.

ACKNOWLEDGEMENTS. This study was partly supported by Grants-in-Aid for Scientific Research (58560283) from Ministry of Education, Science and and Culture of Japan, and Morinaga Foundation.

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要　約

BEK-1 細胞による牛コロナウイルス掛川株のプラック定量法（短報）：平野紀夫・佐田康文・土屋耕太郎・小野勝彦・村上敏明（岩手大学農学部家畜微生物学教室）——BEK-1 細胞における牛コロナウイルス掛川株の吸着は 37℃ 90分で最もよく，寒天培地重層後，37℃ 72時間培養したときにプラック数は最大となり，プラックは明瞭であった。接種ウイルス量とプラック数の間とは相関し，再現性が高く実用的な簡単な定量法が確立された。